Effect of Ethanol Extract of *Phyllanthus niruri* Leaf on Carbon Tetrachloride Induced Hepatotoxicity and Oxidative Stress in Rats

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**ABSTRACT**

The hepatoprotective effect of the ethanol extract of *Phyllanthus niruri* in carbon tetrachloride-induced hepatotoxicity in rat was investigated in this study. The rats were divided into 7 groups; group I (normal control) was given distilled water only, group II rats were administered carbon tetrachloride (CCl$_4$) in olive oil; (1:1 v/v) 3 mL/kg (i.p) once weekly for 28 days (7th, 14th, 21st, and 28th day), while group III, IV and V rats were administered CCl$_4$ as described for group II rats and treated with ethanol extract of *P. niruri* leaves (200, 500 and 1000 mg/kg body weight) once daily from the 7th to 28th day. Group VI rats were given 500mg/kg body weight of the extract only. Fasting blood serum samples and homogenates of livers from the rats were analysed for selected biochemical parameters. Carbon tetrachloride caused increases in serum alanine amino transferase (ALT), aspartate amino transferase (AST), gamma glutamyl transpeptidase (y-GGT) and alkaline phosphatase (ALP). It also caused increases in liver total cholesterol, triglyceride, and malondialdehyde (MDA) levels. Carbon tetrachloride also caused significant reductions in serum total protein, albumin, bilirubin as well as liver superoxide dismutase and catalase activities. The plant extract was able to attenuate all the biochemical parameters to levels comparable to those of the control group. In conclusion, the findings of this study showed that the ethanol leaf extract of *P. niruri* possesses hepatoprotective and antioxidant properties.

**Keys: Hepatoprotective, Antioxidant, Carbon tetrachloride, *P. niruri*.**

**Introduction**

The liver is considered as one of the largest organs in the human body, playing a vital role in the metabolism of carbohydrates, proteins and lipids. The metabolism of xenobiotics to a large extent takes place in the liver and the by-product of such metabolism sometimes becomes more toxic than the parent compound. This could lead to liver damage and ultimately to the emergence of liver disease. The by-products include oxygen containing molecules that are deleterious to cell component through oxidation. The liver contains considerable amounts of polysaturated fatty acids that are prone to free radicals modification and attendant membrane damage.

In the case of chronic alcohol consumption, there are lipid peroxidation, necrosis, and eventually liver damage, accompanied by decreased expression and activity of peroxisome proliferator activated receptor alpha (PPAR), which is involved in fatty acid oxidation, and increased sterol regulatory element binding protein-2 (SREBP-2) and SREBP-1c levels. The latter being transcriptional factors associated with the regulation of cholesterogenic and lipogenic enzymes, respectively, leading to fat accumulation in the hepatocytes. The toxicant carbon tetrachloride (CCl$_4$), is converted by cytochrome P450 oxidase, to trichloromethyl radical (CCl$_3^*$. Trichloromethyl radicals are converted to the more toxic trichloromethyl peroxyl radical (CCl$_3$O$_2^*$) in the presence of oxygen. These metabolites react with polysaturated fatty acids of the cell membrane to abstract hydrogen atom with attendant initiation of lipid peroxidation with attendant reduction in the levels and activities of superoxide dismutase (SOD) and catalase (CAT). Reduced SOD and CAT activities significantly enhance the oxidative stress (OS) status of the liver. The stressed status of the liver cells (parenchymal and nonparenchymal cells), especially activate Kupffer cells and mediate the hepatic inflammatory processes by inducing the production of tumor necrosis factor-$\alpha$ (TNF-$\alpha$) and other cytotoxic cytokines. A previous study showed that the production of these inflammatory factors is associated with the nuclear factor B (NF-$\kappa$ B) pathway and also contributes to increased activating protein 1 (AP-1) expression in liver after CCl$_4$ treatment.

However it is conceivable that plants have intrinsic antioxidant activities which could attenuate CCl$_4$-induced liver damage. One of such plant is *Phyllanthus niruri*. *P. niruri* is a small plant widely distributed in tropical and subtropical regions of Central and South America, India and Indonesia, Africa and the West Indies. It is a herb of Euphorbiaceae family that grows up to 60 cm. Ethnobotanical survey revealed the hepatoprotective activity of this plant and it is validated by some researchers that extracts from the plant have hepatoprotective activities, antioxidant, lipid lowering, anti-diabetic and anti-inflammatory activities. It is based on this background that this study investigated the effect of ethanol extract of *P. niruri* leaf on Carbon tetrachloride Induced Hepatotoxicity and oxidative stress in Rats.

**Materials and Methods**

**Chemicals and Reagents**

The chemicals used were purchased from Sigma (MO, USA). All reagents and chemicals used were of analytical grade.

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Collection of Plant materials
The leaves of Phyllanthus niruri used in this study were collected from an open field at University of Benin, Benin City, Edo state, Nigeria. The fresh plant was identified by Professor M.E. Osawaru of the Department of Plant Biology and Biotechnology of the University of Benin, Benin City, Nigeria.

Extract Preparation
The dried powdered leaves of Phyllanthus niruri (950 g) were extracted with absolute ethanol (5 L) at room temperature for 72 h. The samples were filtered with Whatman No. 50 filter paper and the filtrate evaporated to dryness with a rotary evaporator (RE 300, Bibby Scientific, UK) to give 50 g corresponding to a percentage yield of 0.05%. The residual product was stored in an air-tight container and kept in the refrigerator maintained at 4°C.

Experimental design
Thirty (30) rats of average weight 180 ±10g were used in this study, 5 rats per group.
Group I (control): Rats were administered olive oil (i.p) once weekly for 28 days and allowed free access to water.
Group II: Rats were administered carbon tetrachloride (CCl₄) in olive oil (1:1;v/v; 3 mL/kg b.wt, i.p) once weekly for 28 days (7th, 14th, 21st and 28th day) and allowed free access to water.
Group III: Rats were administered carbon tetrachloride (CCl₄) in olive oil as described for group II rats and treated with ethanol extract of P. niruri leaves (200 mg/kg body weight) once daily from the 7th day to 28th day but allowed free access to water.
Group IV: Rats were administered carbon tetrachloride (CCl₄) in olive oil as described for group II rats and treated with 500 mg of the extract per kg body wt once daily from the 7th day to 28th day but allowed free access to water.
Group V: Rats were administered carbon tetrachloride (CCl₄) in olive oil as described for group II rats and treated with 1000 mg of the extract per kg body wt once daily from the 7th day to 28th day but allowed free access to water.
Group VI: Rats were given 500 mg of the extract per kg body wt once daily from the 7th day to 28th day but allowed free access to water.
On the 28th day, the rats were fasted overnight and sacrificed under chloroform anesthesia on day 29. Blood was collected from the heart, allowed to clot and then centrifuged at 3500 rpm for 10 min. The sera obtained were used for the required biochemical assays. The livers were also excised and a portion of each was homogenized in phosphate buffered saline (PBS). Each homogenate was centrifuged at 4000 rpm for 15 min to obtain supernatant which was also used for the relevant lipid profile and antioxidant assays.

Hepatotoxicity assessment
The hepatic enzymes ALT, AST, γ-GPT and ALP were used as the biochemical indicators of the acute liver injury. ALT and AST were determined by the method of Reitman and Frankel,18 while γ-GPT was determined by the method described by Tietz.19 Serum alkaline phosphatase (SALP) was determined according to the method of Klen et al.17 Total protein and bilirubin were determined using Randox kit based on established methods.19,20

Antioxidant Assay
Lipid peroxidation was assessed by measuring the formation of thiobarbituric acid-reactive substances (TBARS) as described by Aust and buge20. Catalase (CAT) activity was determined according to the method of Asru.21 Superoxide dismutase (SOD) activity was determined according to the method of Misra and Fridovich.22

Histology
Portions of the liver and kidney were fixed in 10% neutral buffered formalin for histology. Thin sections of the liver were dissected and processed using Leica TP2010 automatic tissue processor for 18 h. The processor passed the tissues through fixation, dehydration, dealcosholisation and paraffinification. Ultra-thin sections of 5 µm were sliced from the paraffinned sections using a Thermo scientific semi-automated rotary microtome. The tissues were then subjected to hematoxylin and eosin staining and viewed under a microscope using X 10 and X 40 magnification.

Statistical Analysis
Data were expressed as the mean ± S.E.M of triplicate determinations using the statistical package for social science (SPSS) version 17.0 for windows. Statistical significance was calculated by one-way analysis of variance. Differences between means were estimated by Duncan’s multiple range tests.

Results and Discussion
The effect of ethanol extract of P. niruri leaves in carbon tetrachloride induced hepatotoxicity and oxidative stress in rats were evaluated. Therefore, the present study was designed to validate the ethnomedical use of P. niruri leaves in the treatment of liver diseases.
Administration of CCl₄ caused significant (p ≤ 0.05) increases in ALT, AST and γ-GPT activities when compared to normal control, but the increases in ALP activity were not significant (p > 0.05) when compared to normal control (Table 1). Treatment of the rats with ethanol leaf extract at a dose of 200 mg/kg body weight caused significant (p ≤ 0.05) decreases in ALT and γ-GPT activities when compared to CCl₄ treated group(Table 1, group III). However, there was a non-significant reduction in AST activity when compared to treated group. Similarly, treatment of rats with 500 and 1000 mg/kg of the extract also caused significant (p<0.05) reduction in serum ALT, AST, ALP and γ-GPT levels when compared with CCl₄ intoxicated group in a dose dependent manner (Table 1, group IV and V). Rats exposed to the extract only at a dose of 500 mg/kg (group VI) had significant (p≤0.05) decreased in serum ALT, AST and γ-GPT levels when compared to normal control (Table 1).
Administration of CCl₄ caused significant (p < 0.05) reduction in serum total protein and albumin, but increase in total bilirubin levels relative to control (Fig. 1). However, treatment with the extract at concentrations of 200, 500 and 1000 mg/kg body weight caused significant (p<0.05) increased in total protein and albumin but decreased bilirubin levels in dose dependent manner respectively, relative to group II (CCl₄ only). The rise in serum ALT, AST and ALP, total bilirubin and reduction in total protein and albumin levels in CCl₄ intoxication have been attributed to loss in structural integrity of the liver cell membrane and leakage of these cytoplasmic enzymes into the blood by others.21 In experimental animals carbon tetrachloride undergoes dechlorination caused by cytochrome P-450 dependent mixed function oxidase in the endoplasmic reticulum to form trichloromethyl radical (CCl₃) which combine with cellular lipids and proteins in the presence of oxygen to induce lipid peroxidation.24,26 These interactions alter the structure of the endoplasmic reticulum and other membranes, cause loss of metabolic enzyme activities, reduction of protein synthesis and loss of glucose-6-phosphatase activities, leading to liver injury.27 However, protection of hepatocytes and stabilization of plasma membrane against the damage caused by hepatopoxins is indicated by decreases in the levels of serum transaminases and alkaline phosphatase levels. Also, concurrent depletion of raised bilirubin levels suggests the stability of the biliary function during injury with CCl₄.23
The effects of the extract on total cholesterol and triacylglycerol in carbon tetrachloride exposed rats are presented in Fig.2. Treatment with CCl₄ gave rise to significant (p ≤ 0.05) increases in the concentrations of total cholesterol and triacylglycerol relative to the control. The extract however reduced the cholesterol and triacylglycerol levels when compared to the CCl₄ group only. Exposure of rat to CCl₄ increases the synthesis of triacylglycerol and cholesterol from acetate and also the rate of lipid esterification.28 The increase synthesis of cholesterol by CCl₄ might positively affects the transport of acetate into the liver cell, resulting in an increased substrate availability, thereby lowering β-oxidation of fatty acids, hydroxylation of triglycerides, suppression of
Table 1: Effect of extract on the activities of serum enzymes in CCl₄ exposed rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ALT(U/L)</th>
<th>AST(U/L)</th>
<th>ALP(U/L)</th>
<th>γ-GT(U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (control)</td>
<td>54.00 ± 4.50</td>
<td>64.6 ± 4.80</td>
<td>25.00 ± 2.22</td>
<td>57.57 ± 4.35</td>
</tr>
<tr>
<td>Group II</td>
<td>98.60 ± 1.60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>99.80 ± 1.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.40 ± 2.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>185.34 ± 7.30&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group III</td>
<td>51.00 ± 2.70&lt;sup&gt;b&lt;/sup&gt;</td>
<td>96.25 ± 0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.50 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>114.13 ± 2.70&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group IV</td>
<td>54.75 ± 1.70&lt;sup&gt;b&lt;/sup&gt;</td>
<td>71.75 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.25 ± 2.78&lt;sup&gt;b&lt;/sup&gt;</td>
<td>111.17 ± 2.78&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group V</td>
<td>58.33 ± 1.50&lt;sup&gt;b&lt;/sup&gt;</td>
<td>58.33 ± 0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.33 ± 2.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>103.32 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group VI</td>
<td>23.30 ± 0.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>59.23 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.33 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45.23 ± 0.45&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are Mean ± SEM, n = 5 rats in each group, a as compared with the normal saline (control) group; b as compared with the CCl₄ only group. Group I (Control); Group II (CCl₄ only); Group III (CCl₄ + 200 mg extract); Group IV (CCl₄ + 500 mg extract); Group V (CCl₄ + 1000 mg extract); Group VI (500 mg extract only).

Figure 1: Effect of extract on serum protein and bilirubin levels in CCl₄ exposed rats.
* as compared with the normal saline (control) group; ** as compared with the CCl₄ only group. Group I (Control); Group II (CCl₄ only); Group III (CCl₄ + 200 mg extract); Group IV (CCl₄ + 500 mg extract); Group V (CCl₄ + 1000 mg extract); Group VI (500 mg extract only).

Figure 2: Effect of extract on serum total cholesterol and triacylglycerol in CCl₄ exposed rats.
* as compared with the normal saline (control) group; ** as compared with the CCl₄ only group. Group I (Control); Group II (CCl₄ only); Group III (CCl₄ + 200 mg extract); Group IV (CCl₄ + 500 mg extract); Group V (CCl₄ + 1000 mg extract); Group VI (500 mg extract only).

Figure 3: Effect of extract on antioxidant enzymes activities in rats exposed to CCl₄.
* as compared with the normal saline (control) group; ** as compared with the CCl₄ only group. Group I (Control); Group II (CCl₄ only); Group III (CCl₄ + 200 mg extract); Group IV (CCl₄ + 500 mg extract); Group V (CCl₄ + 1000 mg extract); Group VI (500 mg extract only).

Figure 4: Effect of extract on malondialdehyde levels in rats exposed to CCl₄.
* as compared with the normal saline (control) group; ** as compared with the CCl₄ only group. Group I (Control); Group II (CCl₄ only); Group III (CCl₄ + 200 mg extract); Group IV (CCl₄ + 500 mg extract); Group V (CCl₄ + 1000 mg extract); Group VI (500 mg extract only).
lyosomal triacylglyceride lipase activity and also the content of unsaturated fatty acids, while de novo fatty acid synthesis and saturated fatty acids increases. All this boosts the availability of fatty acids and leads to increased esterification. In CCl₄ exposed rat liver superoxide dismutase (SOD) and catalase (CAT) activities were decreased (Figure 3) and lipid peroxidation as evidenced by malondialdehyde (MDA) level was increased (Figure 4), increased in the CCl₄ administered animals. The SOD and CAT activities are found to be significantly (p ≤ 0.05) increased compared to control group (Figure 3). The elevated level of MDA in CCl₄ treated group was significantly (p ≤ 0.05) reduced when compared to control group (Figure 4). However, treatment of CCl₄ exposed rats with extract at doses of 200 mg/kg bw, 500 mg/kg bw, 1000 mg/kg bw significantly (p ≤ 0.05) increased SOD and CAT activities but a significant (p ≤ 0.05) reduction in MDA levels (Figures 3 and 4).

The body has an effective antioxidant system against free radicals and reactive oxygen species induced damage in which the endogenous enzymatic and non-enzymatic antioxidants such as SOD, CAT and MDA play important roles. The enzymatic antioxidant enzymes work in tandem to protect biological cells from injury/damage. The elevated level of MDA indicates excessive formation of free radicals and activation of lipid peroxidation system resulting in hepatic damage. Histology of liver section of normal control rats showed normal hepatic cells each with well-defined cytoplasm, nucleus and nucleolus and distinct central vein (Figure 5A). The CCl₄ intoxicated rats showed total loss of hepatic architecture with centrilobular hepatic necrosis, fatty changes vacuolization and congestion of sinusoids, kupffer cell hyperplasia, crowding of central vein and apoptosis (Figure 5B). Treatment with CCl₄ exposed rat at doses of 200, 500 and 1000 mg/kg bw of extract showed protective effect against CCl₄ induced damage in a dose dependent manner (Figure 5C-E). The extract at a dose of 500 mg/kg bw showed no evidence of tissue toxicity when administered alone. The result thus obtained is in the agreement with the work carried out by previous researchers. They reported the hepatoprotective activity of methanol extract of P. niruri leaves on CCl₄ induced damage in rat.

Figure 5: Photomicrographs of liver sections. (A) Control rats showing normal liver histology: clear centrioles with well fenestrated sinusoidal space. (B) rats treated with CCl₄ only showing diffuse fatty degeneration and multiple foci of necrosis. (C) rats treated CCl₄ and 200 mg/kg bw P. niruri showing moderate fatty degeneration of hepatocytes and few small foci of necrosis (D) rats treated with 500 mg/kg bw P. niruri and CCl₄ showing mild fatty accumulation although the centriole appears congested. (E) rats treated with 1000 mg/kg bw P. niruri only showing normal hepatocytes and clear centrioles. The liver sections were stained with H/E and observed with a 10 X objective.

Conclusion
The results of this study showed that ethanol leaf extract of P. niruri possesses antioxidant and hepatoprotective activities against CCl₄-induced liver toxicity in rats. Thus, justify the use of the plant in herbal medicine for treatment of liver diseases.

Conflict of interest
The authors declare no conflict of interest.

Authors’ Declaration
The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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